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parent (0) and 10-1 as the paternal parent. The results are very similar to that seen in the 10-1 selfed photograph.

(c) W38 Control and 10-1 x 0 - W38 as previously described. 10-1 x 0 represents 10-1 as the maternal parent and W38 as the paternal parent (0). The results are very similar to that seen in the 10-1 selfed photograph except that in this photo segregation of the herbicide resistance trait can be seen in the 10-1 progeny.

Figure 3 illustrates the effect of Pursuit® postmortem application in tobacco seedling growth, as measured by plant height. C represents the mean of the control (susceptible parent cultivar) plants, while each subsequent bar represents an individual transgenic progeny.

Figure 4 illustrates the effect of the imidazolinone herbicide Pursuit® on AHAS enzymes derived from an *E. coli* transformed with a Trp574 deletion [DEL], a Trp574 substitution [SUB] and an *E. coli* transformed with wild-type [WT] AHAS sequence.

Figure 5 illustrates the effect of the sulfonylurea herbicide Glean® on AHAS enzymes extracted from *E. coli* transformants. Abbreviations as in Figure 4.

Figure 6 illustrates the effect of the imidazolinone herbicide Pursuit® on AHAS enzymes extracted from *E. coli* transformants. The enzymes are derived from an *E. coli* transformed with a Ser653 deletion [DEL], an *E. coli* transformed with a Ser653 substitution [SUB], and an *E. coli* transformed with a wild-type [WT] AHAS sequence.

Figure 7 illustrates the effect of the sulfonylurea herbicide Glean® on AHAS enzymes extracted from *E. coli* transformants. Abbreviations as in Figure 6.

Figure 8 illustrates the effect of the imidazolinone herbicide Pursuit® on an AHAS enzyme derived from a tobacco plant with an *Agrobacterium* strain containing a mutant *Arabidopsis* AHAS allele having the Trp574 deletion.

DETAILED DESCRIPTION OF THE INVENTION

The entire nucleotide sequence of the AHAS gene in a number of organisms including yeast, *E. coli*, Brassica, *Arabidopsis*, sugar beet and tobacco has been previously disclosed (Mazur et al., supra; Lee et al., EMBO J. 7:1241, 1988). Moreover, it has been noted that herbicide resistance is associated with one or more mutations within the AHAS sequence; specifically, resistance has been noted to be associated with certain specific amino acid substitutions within the sequence. (EP 258 783; Yadav et al. PNAS USA 83:4418-22, 1986; Sathasivan et al., Nucl. Acids res. 18: 2188, 1990). It has, however, been implied (Hartnett et al., in Managing Resistance To Agrichemicals, Ch. 31, pp. 459-473, American Chemical Society, 1990; EP 258 783) that these regions of conservation in which mutations conferring resistance occur must generally be maintained to conserve enzymatic function.

It has now been unexpectedly discovered that conservation of these sequences is apparently not essential to the catalytic function of the molecule. In the course of development of the present invention, site directed mutagenesis of the *Arabidopsis* AHAS coding region is utilized to create deletion mutations in which a residue which has previously been shown to be substitutable is then deleted from the sequence. Deletion of a residue clearly destroys the conserved nature of the regions in which they occur. Nonetheless, the deletion mutations thus created all retain AHAS function, while also exhibiting herbicide resistance. Specifically, single deletions of the Trp574, Pro197 and Ser653 residues and double deletions of Pro197 and Ser653 are made to create fully functional, herbicide resistant plants. All the numbering of amino acids herein is based on the *Arabidopsis* AHAS sequence. However, it will be understood that, throughout the specification and claims, reference to a specific site of deletion in the *Arabidopsis* sequence is meant to encompass the corresponding sites in the AHAS sequence of any other species having an AHAS gene.

In view of these data, it becomes apparent that conservation of those herbicide-resistance associated regions of the AHAS molecule is not critical for catalytic activity, and that one or more amino acid deletions in one or more "conserved" amino acid sequences can easily be tolerated by the enzyme and further, will confer herbicide resistance to the plant. The present invention encompasses AHAS DNA sequences in which one or more deletions have been made relative to the wild-type sequence, which deletions do not alter the catalytic function of the resulting AHAS enzyme, but which confer herbicide resistance. Such deletion mutations include, but are not limited to, deletion of one or more codons, encoding the so-called "conserved subsequences" as defined in EP 257 783. These are DNA sequences encoding amino acids 119-122, 184-187, 201-208, 255-257, 348-353, 373-377, and 589-578 in *Arabidopsis* AHAS. Additionally, a further "conserved sequence" in which substitution has been described and in which deletion is useful in bringing about herbicide resistance, is 650-653. Deletion of one codon, more than one codon, and up to all the codons in the "subsequences" noted above, is considered within the scope of the invention. Preferred

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are codon deletions encoding mutants having a deletion of at least one residue position selected from the group consisting of amino acid 120, 121, 197, 205, 256, 351, 378, 571, 574, 578 and 653. Particularly preferred are sequences encoding deletions at 197, 574 or 653.

Although the above-identified deletions represent regions in which variation is known to be tolerated, it is likely, in view of the substantial "flexibility" of the molecule that other deletions would also be feasible. For example, other apparently "conserved" regions of the AHAS enzyme also exist in addition to those outlined above. While not wishing to be bound by any particular theory, it now appears likely that, rather than being sites connected with catalytic activity of the molecule, and therefore requiring essential structural conservation, most of these conserved regions represent binding sites for the herbicide. Deletion of one or more amino acids at these sites would then logically prevent herbicide binding, and consequently, prevent herbicide interference with AHAS activity. Using this rationale, the available knowledge regarding "conserved" AHAS sequences (See, e.g., Mazur et al., *Plant Physiol.* 85: 1110-1117, 1987) and known techniques, such as site-specific mutagenesis, it would be a routine matter to design additional deletion mutants likely to have herbicide resistance. Putative mutants can then be screened by growth in the presence of inhibitory amounts of the herbicide of interest, to determine whether or not herbicide resistance has been conferred.

As with all proteins, the functional AHAS enzyme possesses a three-dimensional structure which is the eventual result of the linear arrangement of the component amino acids. Interaction of the side groups of amino acids produces a protein's secondary structure, and further folding results in a protein's tertiary structure. A given protein's specific "architecture" which ultimately results from the amino acid sequence can be critical to the protein's function. Thus, the substitution of an amino acid retains the overall structural integrity of the protein, whereas the deletion of an amino acid effectively destroys this structural integrity and can be expected to significantly alter the protein's three-dimensional structure and, in doing so, alter the function of the molecule. Therefore, in view of the potential damage that can be caused by a deletion, it is particularly surprising that the resultant molecule retains sufficient portions of its three dimensional structure to remain not only catalytically functional, but also to cause herbicide resistance.

Those skilled in the art will recognize that the deletion mutants of the present invention are not limited as to the source of the DNA or enzyme. Although the present experimental design primarily utilizes the *Arabidopsis* AHAS gene sequence, similar mutations can routinely be made in the AHAS sequence of any organism possessing such a gene, e.g. other higher plants, yeast, *E. coli*, and other microorganisms. The similarities in the wild-type AHAS gene among all the known sequences are so great as to render it a matter of routine experimentation to create identical mutants in AHAS sequences other than that of *Arabidopsis*. Alternately, chimaeric genes can be constructed which contain the deletion mutant portion of the *Arabidopsis* AHAS gene recombined with unaltered portions of the AHAS gene from other sources.

The novel gene types described herein may confer resistance to one, or more than one, type of herbicide. As has already been well established, AHAS is the site of action of several distinct classes of herbicides, namely, imidazolinones, sulfonylureas, triazolopyrimidines, sulfamoylureas and sulfonylcarboxamides. As with herbicide resistance conferred by amino acid substitution, the herbicide resistance conferred by such mutations may be selective to a particular herbicide, or there may be cross-resistance to more than one herbicide. For example, deletions of Trp574 and Ser653 create cross-resistance to both imidazolinones and sulfonylureas. One skilled in the art can, however, easily determine the specificity of any particular mutant by screening separately in the presence of, e.g., imidazolinones only, or sulfonylureas only, and isolating surviving plants. Cross resistance can be determined by growth in the presence of more than one class of herbicide. Types of herbicides with which the present invention is useful are described, for example, in U.S. Patent No. 4,188,487; 4,201,565; 4,221,586; 4,297,128; 4,554,013; 4,608,078; 4,638,068; 4,647,301; 4,650,514; 4,688,092; 4,701,208; 4,709,038; 4,752,323; 4,772,311; and 4,788,610; U.S. Pat. Nos. 4,127,405; 4,435,206; 4,424,703; 4,417,917; 4,388,838; 4,394,506; 4,391,627; 4,383,113; 4,378,891; 4,372,778; 4,371,391; 4,370,480; 4,370,479; 4,369,320 (sulfonylureas).

At this time, AHAS has been demonstrated to be not only present in a wide variety of plants, but has also been shown to be a critical site determining herbicide sensitivity in a broad range of essentially unrelated plants, e.g. corn, *Brassica*, tobacco, flax, *Arabidopsis*, and sugar beet (Stougaard et al., *Mol. Gen. Genet.* 219: 413-420, 1989; Jordan & McHughen, *J. Plant Physiol.* 131: 333-338, 1987; McHughen, *Plant Cell Reports* 8: 445-449, 1989). As noted above, then, it is possible to create the relevant mutation directly in the plant of interest by known mutagenic techniques. (See, for example, Maniatis, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982; and Example II, *infra*). However, it will frequently be more convenient to create transformed plants with a known and isolated DNA sequence comprising the requisite deletions, such as the *Arabidopsis* deletion mutants described in the present case. Plasmids containing deletion mutants at positions 574 and 653 have been deposited with the American

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Type Culture Collection, Rockville, MD, on December 6, 1990 as, respectively, accession numbers ATCC 68489 and 68489.

Isolated AHAS DNA sequences of the present invention are useful to transform target crop plants, and thereby confer herbicide resistance without the necessity for mutagenesis. A broad range of techniques currently exist for achieving direct or indirect transformation of higher plants with exogenous DNA, and any method by which the novel sequence can be incorporated into the host genome, and stably inherited by its progeny, is contemplated by the present invention. A detailed description of one such method is provided in the following examples.

Indirect transformation of plant cells can be achieved by the use of vectors. A common method of achieving transformation is the use of *Agrobacterium tumefaciens* to introduce a foreign gene into the target plant cell. For example, in the present case, the mutant AHAS sequence is inserted into a plasmid vector containing the flanking sequences in the Ti-plasmid T-DNA. The plasmid is then transformed into *E. coli*. A triparental mating among this strain, an *Agrobacterium* strain containing a disarmed Ti-plasmid containing the virulence functions needed to effect transfer of the AHAS containing T-DNA sequences into the target plant chromosome, and a second *E. coli* strain containing a plasmid having sequences necessary to mobilize transfer of the AHAS construct from *E. coli* to *Agrobacterium* is carried out. A recombinant *Agrobacterium* strain, containing the necessary sequences for plant transformation is used to infect leaf discs. Discs are grown on selection media and successfully transformed regenerants are identified. The recovered plants are resistant to the effects of herbicide when grown in its presence. Other plant vectors, such as plant viruses, also provide a possible means for transfer of exogenous DNA.

Direct transformation of plant cells, instead of the use of vectors, can also be employed. Typically, protoplasts of the target plant are placed in culture in the presence of the DNA to be transformed, and an agent which promotes the uptake of DNA by protoplast is absorbed on their surfaces. Useful agents in this regard are polyethylene glycol or calcium phosphate.

Alternatively, DNA uptake can be stimulated by electroporation. In this method, an electrical pulse is used to open temporary pores in a protoplast cell membrane, and DNA in the surrounding solution is then drawn into the cell through the pores. Similarly, microinjection can be employed to deliver the DNA directly into a cell, and preferably directly into the nucleus of the cell.

In each of the foregoing techniques, transformation occurs in a plant cell in culture. Subsequent to the transformation event, plant cells must be regenerated to whole plants. Techniques for the regeneration of mature plants from callus or protoplast culture are now well known for a large number of different species (see, e.g., Handbook of Plant Cell Culture, Vols. 1-5, 1983-1988 McMillan, N.Y.) Thus, once transformation has been achieved, it is within the knowledge in the art to regenerate mature plants from the transformed plant cells.

Alternate methods are also now available which do not necessarily require the use of isolated cells, and therefore, regenerative techniques, to achieve transformation. These are generally referred to as "ballistic" or "particle acceleration" methods, in which DNA coated metal particles are propelled into plant cells by either a gunpowder charge (Klein et al., Nature 327: 70-73, 1987) or electrical discharge (EPO 270 356). In this manner, plant cells in culture or plant reproductive organs or cells, e.g. pollen, can be stably transformed with the DNA sequence of interest.

The present invention can be applied to transformation of virtually any type of plant, both monocot and dicot. Among the crop plants for which transformation to herbicide resistance is contemplated are corn, wheat, rice, millet, oat, barley, sorghum, alfalfa, sugar beet, Brassica species, tomato, pepper, soybean, tobacco, melon, squash, potato, peanut, pea, cotton, or cacao. The novel sequences may also be used to transform ornamental species, such as rose, and woody species, such as pine.

The novel sequences of the invention also is useful as selectable markers in plant genetics studies. For example, in plant transformation, sequences encoding herbicide resistance could be linked to a gene of interest which is to be used to transform a target herbicide - sensitive plant cell. The construct comprising both the gene of interest and the herbicide resistant sequence are introduced into the plant cell, and the plant cells are then grown in the presence of an inhibitory amount of the herbicide. Plant cells surviving such treatment presumably have acquired the resistance gene as well as the gene of interest, and therefore, putative transformants are readily identifiable.

The invention is further illustrated by the following non-limiting examples.

65 EXAMPLE I

Isolation of a Genomic Clone Encoding Arabidopsis AHAS

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A 2.1Kb EcoRI fragment encompassing the promoter, transit peptide and a portion of the mature coding region of Arabidopsis AHAS is 32 P-labelled by nick translation (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982) and utilized as a hybridization probe to screen a genomic library prepared from Arabidopsis thaliana genomic DNA (Clontech, Palo Alto, CA). The filters are hybridized for 24 hr. at 42°C in 6xSSC, 5 x Denhardt's solution, 50mM sodium phosphate, pH7.2, 0.1% SDS and 100ug/ml of denatured salmon sperm DNA. Filters are washed several times at 60°C in 1xSSC and 0.1% SDS. Six recombinant bacteriophage (A22, A31, A42, A52, A72, A83) were classified as putative positives. These bacteriophage are plaque purified and infected at low multiplicity with the *E. coli* strain K802 in liquid NZY broth (NZCYM is 10 g NZ amine, 5 g NaCl, 5 g yeast extract, 1 g casamino acids, 2 g MgSO₄·7H₂O and 10ml of 1M Tris-HCl, pH7.2) as described by Maniatis, et al, supra. Liquid cultures are incubated overnight with constant shaking (300 rpm) at 37°C. The 50ml suspension is brought to 1M NaCl and 8% PEG by the addition of solid NaCl and solid polyethylene glycol. The suspension is incubated overnight at 4°C to precipitate the bacteriophage. The bacteriophage are pelleted via centrifugation at 18,000 x g for 20min. The pellet is resuspended in 2ml of TM buffer (10mM Tris-HCl, pH 7.5; 10mM MgCl₂), layered upon a CsCl step gradient (4.8M CsCl, 4.0M CsCl and 3.2M CsCl) and centrifuged at 50,000 x g for 1 hr in a SW60 swinging bucket rotor. The phage band is removed from the 4.0/3.2M CsCl interface to a 1.5ml Eppendorf tube and the bacteriophage lysed by adding 1 volume of formamide and incubating at room temperature for 30 minutes. DNA is recovered by bringing the solution to 10m MTris-HCl, pH 8.0 and 1mM Na₂-EDTA and precipitating the DNA via the addition of 2 volumes of 100% ethanol. Bacteriophage DNA is recovered via centrifugation, washed with 70% ethanol and resuspended in TE buffer (TE buffer is 10mM Tris-HCl, pH 7.5; 1mM Na₂-EDTA). The purified DNA is routinely extracted one to several times with phenol:chloroform:isoamyl alcohol (24:24:1), ethanol precipitated, and resuspended in 50-100ul of TE buffer prior to digestion with various restriction enzymes.

DNA preparations from the six recombinant phage are digested with various restriction enzymes and resolved by electrophoresis through a 1% agarose gel. The DNA is transferred to nitrocellulose (Soulhorn) and hybridized (24 hr. at 60°C in 2xSSC, 5 x Denhardt's solution, 50mM sodium phosphate, pH7.2, 0.1% SDS and 100ug/ml of denatured salmon sperm DNA) against a radiolabeled 800 bp NcoI/EcoRI fragment representing the 3' region of the 2.1Kb EcoRI fragment (the transit peptide and the 5' portion of the mature coding region). The filter is washed twice at 65°C in 0.5xSSC and 0.1% SDS. The 800 bp NcoI/EcoRI fragment hybridizes to a 5.5Kb XbaI and 2.1 EcoRI fragment of both A42 and A52. These data are in agreement with published information concerning the cloning and characterization of the genomic sequence encoding Arabidopsis AHAS (Mazur et al., Plant Physiol, 85: 1110-1117, 1987).

The bacteriophage A52 is chosen for further analysis. The 5.5 Kb XbaI fragment of A52 is isolated from a 1% agarose gel and purified through an IBI electroelution device as suggested by the manufacturer. The 5.5Kb XbaI fragment is ethanol precipitated and resuspended in ddH₂O. These fragments are ligated to XbaI digested-pSK(-) plasmid DNA (purchased from Stratagene, La Jolla, CA). The fragments are ligated for 16-24 hours at 14°C in a 20ul volume as described by Maniatis, et al., supra. Following incubation, the 20ul reaction volume is diluted to 100ul with 100mM Tris-HCl, pH7.2. Twenty five ul of this sample is diluted further to 100 ul with 100mM Tris-HCl, pH7.2 and incubated with 100ul of competent HB101 cells [prepared exactly as described by Morrison (Meth. Enzymol. 68: 326 - 331, 1979)]. The plasmid/*E. coli* transformation mix is incubated on ice for 20 minutes; transferred to 42°C for 2 minutes; room temperature for 10 minutes; followed immediately by the addition of 1 ml of LB broth (LB broth is 10 g bacto-tryptone; 5 g yeast extract; 5 g NaCl and 10 ml 1 M Tris-HCl, pH 7.2 per liter) and further incubation at 37°C for 20 minutes. DNA plasmid cloning vehicles used in this work carry either the gene conferring resistance to ampicillin or kanamycin. Therefore, 200 ul of each transformation mix is plated directly onto either LB_{amp} or LB_{kan} plates (100 ug/ul of either respective antibiotic) and incubated at 37°C for 12-20 hr, depending on colony size.

Colonies are transferred to 3ml of LB_{amp} or LB_{kan} and incubated overnight at 37°C with constant shaking. Small scale plasmid DNA preparations are prepared by minor modifications of the alkaline lysis method described by Maniatis, et al. supra. Routinely, 400 ul of the 3ml overnight culture is pelleted in a 1.5ml Eppendorf tube and resuspended in 150 ul of GTE solution (GTE solution is 50mM glucose; 25mM Tris-HCl, pH 8.0 and 10mM Na₂-EDTA) and incubated on ice for 20 minutes, followed by the addition of 300 ul alkaline-SDS solution and 200 ul of 5M K⁺/3M OAc at the prescribed time intervals. The final DNA pellet is resuspended in 40ul of 50ug/ul RNase A in TE buffer. Fifteen ul of plasmid DNA is removed for restriction digestion. Two plasmids, pAC301 (5'→3') and pAC302 (3'→5'), represent constructions containing the 5.5Kb XbaI fragment ligated to the XbaI site of pSK(-). Further restriction analysis of pAC301 and pAC302 confirm the presence of the promoter region, transit peptide, mature coding region and 3'non-

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translated regions of the genomic sequence encoding *Arabidopsis* AHAS (Mazur et al., *Supra*).

EXAMPLE II

6 Oligonucleotide site directed mutagenesis of the *Arabidopsis* AHAS coding region

(a) Preparation of single stranded template of pAC301 - The plasmid pAC301 is transformed into the *E. coli* strain, XL-1 (Stratagene). Single stranded template of pAC301 is generated from this pSK(-) "phagemid" based construction by following the manufacturers protocol. Routinely, a 3ml SB_{amp} overnight culture is added to a sterile flask containing 50ml of SB_{amp} (SB is 35 g bacto-tryptone, 20 g yeast extract, 5 g NaCl and 10 ml of 1M Tris-HCl, pH 7.5 per liter) and incubated at 37°C with constant shaking (300 rpm) until the culture reached an OD₆₀₀ of 0.3. At this point the culture is inoculated with the helper phage, R408 (1×10^{10} phage) and vigorous 10° shaking at 300 rpm at 37°C was continued overnight. The bacteria are pelleted via centrifugation at 10,000 x g for 10 minutes. The bacterial pellet is discarded and the supernatant (approximately 45 ml) containing the single stranded pAC301 template is stored at 4°C until use. The supernatant is routinely used for up to one month for single stranded phagemid preparations. Small scale preparations (1.2ml) of single stranded pAC301 phagemid DNA are prepared essentially as described by the manufacturer (Stratagene). The phage are precipitated by adding 300 ul of a 3.5M NH₄Ac, pH 7.5; 20% PEG solution. The solution is mixed well in a 1.5 ml Eppendorf tube and incubated at room temperature for 20 minutes. The single stranded phage are pelleted by centrifugation for 20 minutes in an Eppendorf microcentrifuge. The supernatant is decanted, the pellet dried and eventually resuspended in 300 ul of TE buffer. The phage are lysed by adding 200 ul of phenol:chloroform:isoamyl alcohol (24:24:1) and vortexing for 1 minute. This step is repeated once more and followed by two more extractions with chloroform:isoamyl alcohol (24:1). The final aqueous phase is ethanol precipitated (one-tenth volume 5M K₂SO₄ and two volumes of 100% ethanol), washed with 70% ethanol, resuspended in 20ul of TE buffer and transferred to a fresh 1.5ml Eppendorf tube.

(c) Preparation of the oligonucleotide - All oligonucleotides are purchased from New England Biolabs. Oligonucleotides are utilized to introduce (1) an amino acid substitution at the Trp574 residue of the *Arabidopsis* AHAS coding region (Trp → Leu) and (2) an amino acid deletion at the Trp574 amino acid residue.

Trp574 → Leu substitutions

35
V N Q W E D R
wild type 5'-GTT ATG CAA TGG GAA GAT CGG-3'
V N Q L E D R
mutant 5'-GTT ATG CAA TTG GAA GAT CGG-3' (21-mer)

Trp574 deletion

45
V N Q W E D R
wild type 5'-GTT ATG CAA TGG GAA GAT CGG-3'
V N Q E D R
mutant 5'-G GTT ATG CAA --- GAA GAT CGG- 3'
(21-mer)

Two hundred nanograms of each oligonucleotide is subjected to a kinase exchange reaction prior to hybridization to pAC301 single stranded template. A 40 ul reaction volume included 50mM Tris-HCl, pH7.5; 10mM MgCl₂, 50mM DTT, 0.1mM spermidine and 0.1mM Na₂-EDTA. The reaction is initiated via the addition of 10 units of T4 polynucleotide kinase (Pharmacia) and is incubated at 37°C for 30 minutes. The reaction is terminated by bringing the reaction mixture to 80°C for 3 minutes. One-half of the kinase reaction is added to the entire 20 ul pAC301 single stranded prep and is incubated at 65°C for 10 minutes to promote hybridization. To this 40 ul kinase/oligonucleotide mix is added 6 ul of 1mM dNTP's, 6 ul of 10 x

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ligase buffer (500mM Tris-HCl, pH7.5; 70 mM MgCl₂ and 10mM DTT), 2 ul 10mM rATP, 5 units of Klenow DNA polymerase (Pharmacia), 8 units of DNA ligase (Stratagene) and 4ul ddH₂O. The polymerase/ligation reaction is incubated at room temperature for 3 hr. One-half of the mix is used to transform competent *E. coli* XL-1 cells. The transformation mix is spread to LB_{amp} plates and incubated overnight at 37°C.

5 Transformants are restreaked to fresh LB_{amp} plates in a grid fashion. Colony screening by hybridization was performed exactly as described by Stratagene Technical Service (June 1988/pBBII Exo/Mung Bean DNA Sequencing System). Both oligonucleotides are ³²P-labeled by a kinase exchange reaction (300 ng of oligonucleotide in the presence of 40 uCi of ³²P-ATP utilizing kinase conditions described previously). Unincorporated ³²P-ATP is removed by electrophoresing the kinase reaction through a 20% acrylamide/7M

10 urea gel. The radioactive band corresponding to the 21-mer oligonucleotide is cut out of the gel with a razor blade and eluted either by (1) the crush and soak method of Maxam and Gilbert (PNAS USA 74: 560-568, 1977) or (2) electroelution through an IBI electroelution device. The purified, radiolabeled oligonucleotides are hybridized to the nitrocellulose colony lifts under non-stringent hybridization conditions (37°C for 24 hrs. in 6xSSC, 5 x Denhardt's, 50mM Na-P, pH7.2 and 500 ug/ul of calf thymus DNA). Filters are washed once

15 at room temperature in 6xSSC and 50 mM Na-P, pH 7.2 and again at 37°C in 6xSSC and 50mM Na-P, pH 7.2. Finally, the filters are washed twice at 60°C for 15 minute intervals in 3M tetramethylammonium chloride, 50mM Tris-HCl, pH 7.5, 2mM Na₂-EDTA and 1 mg/ml of SDS. A 21-mer oligonucleotide with a perfect base to base hybridization match (i.e., mutant oligo to mutant phagemid) is not washed free of the phagemid DNA at 60°C, whereas a slight mismatch (i.e., mutant oligo to wild type phagemid) is washed

20 free. Putative positives are streaked from the original LB_{amp} plate to a fresh LB_{amp} plate. Ten transformants from each restreaked putative positive are then restreaked in a grid pattern and the colony hybridization is repeated. Secondary positives are then confirmed via DNA sequence analysis through the Trp574 coding region. (Sanger et al., PNAS USA 74: 5463-5467, 1977). Two positive mutants, pAC324 (Trp574 → Leu substitution) and pAC325 (Trp574 deletion) were selected for further analysis.

EXAMPLE III

Agrobacterium Mediated Transformation of Tobacco

30 (a) Construction of vectors - Both vectors and the *Agrobacterium* strain are purchased from Clontech. The plasmid pBIN19 is an *E. coli*/Agrobacterium binary shuttle vector that possesses both the left and right border sequences of the Ti-plasmid T-DNA, a polylinker, an RK2 bacterial origin of replication functional in both *E. coli* and Agrobacterium, and a gene conferring resistance to kanamycin (Bevan, Nucl. Acids Res. 12: 8711-8721, 1984). The plasmids pAC324 and pAC325 are digested with XbaI and electrophoresed through a 1% agarose gel. The 5.5Kb XbaI fragment containing the respective mutation of

35 pAC324 and pAC325 are isolated as previously described. The plasmid pBIN19 is digested with XbaI and incubated in a separate ligation reaction with the 5.5Kb XbaI fragment from pAC324 and pAC325. The entire ligation mix is transformed into *E. coli* XL-1 cells and positive transformants are chosen via a blue/white color selection on LB_{amp} plates (LB plates with 100 ug/ul ampicillin, 80 ug/ml X-Gal and 20mM IPTG). Small

40 scale plasmid preparations, restriction digestion analysis and agarose gel electrophoresis to confirm positive transformants are performed as described previously. Of the four pBIN19 based plasmid constructions, pAC348-pAC349 (both orientations of the 5.5Kb XbaI fragment containing the Trp574→Leu substitution in pBIN19) and pAC350-pAC351 (both orientations of the 5.5Kb XbaI fragment containing the Trp574 deletion in pBIN19), one construction from each respective mutation is chosen for transformation of tobacco.

(b) Mobilisation of pAC348 - pAC351 into Agrobacterium

The plasmid pRK2013 is a conjugative plasmid that contains trans acting sequences required to mobilize pBIN19 based constructions from *E. coli* into the disarmed Agrobacterium strain, LBA4404 (pAL4404). This Agrobacterium strain is resistant to streptomycin and contains the disarmed Ti plasmid

50 pAL4404 (Ooms et al., Plasmid 7: 15-28, 1982). This Ti plasmid contributes the transacting virulence functions necessary to facilitate transfer of the pBIN19 based T-DNA region into the chromosome of tobacco. The triparental mating of the pBIN19 based vectors, the conjugative plasmid (pRK2013), and the Agrobacterium strain (LBA4404), are carried out essentially as described by Bevan (Nucl. Acids Res. 12: 8711-8721, 1984). The plasmids pAC348-351 (Kan^r) and pRK2013 (Kan^r) are transformed into competent *E. coli* HB101 or XL-1 cells as described previously. Transformants containing each plasmid are inoculated into 3ml LB_{amp} broth cultures and incubated at 37°C with constant shaking. The Agrobacterium strain, LBA4404, is inoculated into AB_{amp} media (20 x AB is 20g NH₄Cl, 8g MgSO₄·7H₂O, 3g KCl, 60g K₂HPO₄,

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20g NaH_2PO_4 , 3g CaCl_2 and 50mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Glucose is added to a 1x stock to a final concentration of 0.5x and incubated with constant shaking at 28°C for 36-48 hr. The triparental mating is initiated by combining 1 ml of each of the three cultures (a pBIN19 based construct, pRK2013 and LBA4404) into a sterile tube and continuing incubation at 28°C for 1 hr. The culture is concentrated via vacuum onto a 0.45µM filter and incubated overnight at 28°C on an NB plate (NB is 4g of Difco nutrient broth powder and 10ml of 1M Tris-HCl, pH 7.2). The filter is placed in 2ml of fresh AB media and rotated slowly for 1 hr. A dilution series is spread onto $\text{AB}_{\text{strp}^{\text{kan}}}$ plates and incubated at 28°C for 3-4 days. Only *Agrobacterium* - (strp^r) carrying the pBIN19 based construct (kan^r) grow on $\text{AB}_{\text{strp}^{\text{kan}}}$ media. Single colonies from $\text{AB}_{\text{strp}^{\text{kan}}}$ plates are inoculated into $\text{AB}_{\text{strp}^{\text{kan}}}$ broth and incubated at 28°C with constant shaking for 36-48 hr. These cultures are restreaked to $\text{AB}_{\text{strp}^{\text{kan}}}$ plates, incubated at 28°C for 3-4 days and stored at 4°C until use.

(c) *Agrobacterium*-Mediated Transformation of Tobacco

pAC348 and pAC351 are inoculated into 50ml of AB_{kan} and incubated with constant shaking at 28°C for 36-48 hr. Bacteria are pelleted by centrifugation at 2500 rpm for 10 minutes in a Damon/MEC table top centrifuge. The bacterial pellet is resuspended in 5ml of BAT media (BAT media is 1x MS salts (Murashige and Skoog, Plant Physiol. 15: 473-487, 1962) 1x B₅ vitamins (100x B₅ is 10mg myo-inositol, 100mg nicotinic acid, 100mg of pyridoxine-HCl and 1000mg Thiamine-HCl per 1 liter), 3% sucrose, 5µM 6-benzylaminopurine at pH 5.7). Young greenhouse grown Wisconsin 38 tobacco leaves are cut into 2 longitudinal sections and soaked in warm water containing Ivory hand soap. The leaf sections are washed in sterile distilled water, soaked in a 10% Clorox + Tween 20 solution for 10 minutes with stirring and rinsed 3x with ddH₂O. Discs are cut from the leaf sections using either a cork borer or hole-punch. These discs are placed in a 50ml tube containing the resuspended *Agrobacterium* culture. The suspension is mixed gently for 5 minutes and blotted onto a sterile paper towel and cultured onto BAT media plates (100 x 20mm plates with approximately 10 discs per plate). The plates are incubated at 25°C under fluorescent lighting for 48hr. The discs are then transferred to selection media (BATCK; BAT media plus carbenicillin and kanamycin) and returned to the initial incubation conditions until the formation of shoots. Kanamycin resistant shoots are transferred to OTCK (rooting) selection media (OTCK media is BATCK media minus 6-benzylaminopurine) in (GA7) Magenta boxes and the previously described incubation conditions were continued. When kanamycin resistant shoots form an appreciable root system (at least 3 roots with lengths longer than 1cm), the plants are transferred to soil. Briefly, the agar based shoot is removed from the GA7 box, the agar is carefully washed away with warm tap water and the shoots are transferred to metromix in peat pots in GA7 vessels. The plants are placed in the greenhouse and allowed to harden off. A total of 5 kanamycin resistant shoots containing pAC348 and 8 kanamycin resistant shoots containing pAC350 are transferred to soil. Approximately 10-14 days later, the peat pots are transplanted to larger pots.

pAC348 Transformant #:	pAC351 Transformant #:
1) 10-1	1) 10-1
2) 33-1	2) 20-1
3) 33-2	3) 20-2
4) 46-1	4) 20-3
5) 46-2	5) 27-1
	6) 27-2
	7) 27-3
	8) 30-1
	9) 42-4

EXAMPLE IV

Determination and Characterization of Mutants Conferring Herbicide Resistance

AHAS enzyme assay (leaf tissue) - Shortly after transfer to the greenhouse, extracts each of the kanamycin resistant transformants are prepared and assayed for insensitivity to the imidazolinone Pursuit® herbicide. Acetohydroxyacid synthase is extracted and assayed as described in the previous section. Two kanamycin resistant transformants (33-1 and 46-2) which contain the Trp574→Leu substitution mutant allele of the Arabidopsis AHAS allele exhibit AHAS activity in the presence of Pursuit® herbicide. In addition,

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AHAS activity of one kanamycin resistant deletion transformant (10-1) exhibits insensitivity to the addition of Pursuit® herbicide (Figure 7). This Trp574 deletion transformant is selfed and backcrossed to Wisconsin 38. Progeny from 10-1 exhibit tolerance to post-emergence applications of Pursuit® herbicide 4-8x greater than the concentration lethal to control tobacco plants.

Figures 2(a)-(c) show photographic comparisons of the phenotypes of the crosses and controls discussed above. These observations indicate inheritance of resistance observed in the initial AHAS assay from transformed leaf tissue, and expression of this trait at the whole plant level.

In a more quantitative assay, seed of selfed progeny of the transgenic tobacco plant (10-1) containing the Trp574 deletion mutation of Arabidopsis AHAS as well as seeds of the susceptible parental cultivar "Wisconsin 38" are planted in Metro Mix 350™ in 5" Azalea pots. The seedlings are thinned to the single most vigorous seedling after 2 days. Eleven days later, these plants are sprayed postemergence with Pursuit® at 0, 10, 20, 40, 80 and 160 g/ha. Five plants of each tobacco type (transgenic and control) are sprayed per herbicide rate. Tween 20™ is added at 0.25 v/v to the herbicide solutions prior to spraying. The herbicide is applied with a laboratory belt sprayer at a rate of 400 L/ha at a distance 18" above the plants with a belt speed of 8.2 sec/rev and sprayer nozzle #65015E. Plant heights are measured 1, 2, 3, and 4 weeks after treatment, plant fresh weight data is collected at four and one-half weeks.

The results of these sprayings are depicted in Figure 4. The progeny data in the graph are presented with the most tolerant individual first and the most susceptible individual last for each herbicide rate. As can be seen from this graph and as would be expected from a selfed progeny of the original transgenic plant, the progeny are segregating for herbicide tolerance. Both susceptible progeny and individuals with varying degrees of herbicide tolerance can be observed.

The plant fresh weights (g) and means for each herbicide rate are presented in Table 2 with the mean fresh weights (g) summarized below (Table 1):

Table 1

Tobacco Mean FW: (5 reps each)	Control	Transgenic Progeny
Herbicide Rate:		
0 g/ha	52.1	53.7
10 g/ha	6.1	36.4
20 g/ha	2.0	28.8
40 g/ha	0.8	20.9
80 g/ha	0.6	26.5
160 g/ha	0.2	21.4

As can be seen in this table, the selfed progeny of the transgenic tobacco plant (10-1) exhibit a high degree of tolerance to Pursuit® applied postemergence. The susceptible parental cultivar does not display herbicide tolerance at any of the rates tested.

Seeds resulting from transgenic plant 10-1 are also assayed for resistance to the imidazolinone Pursuit®. Seeds are plated onto medium containing Pursuit® at 0, 1.25, 2.5, 3.75, and 5.0 µM. Twenty seeds are plated per petri dish, with two dishes per treatment. Herbicide tolerance of seedlings is evaluated after three weeks. The results of the 5.0 µM treatment is presented in Table 2. Even at the lowest concentration (1.25 µM treatment), control plants exhibited herbicide sensitivity. These results illustrate inheritance of the herbicide resistant trait from parental plant 10-1 to its progeny.

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Table 2

Results of tobacco seed assay				
Pursult® Conc.(μM): 5		S*	D	R
Progeny:	W38 (wild-type)	20	0	0
		20	0	0
	10-1 Selfed	7	6	6
		3	11	6
	10-1 x 0	11	9	0
		11	9	0
	0 x 10-1	10	9	0
		8	8	0

* S = Susceptible; D = Damaged; R = Resistant

20 EXAMPLE V

AHAS enzyme assay in *E. coli*

AHAS enzyme assay (expression in *E. coli*) - The site directed mutants, pAC224 and pAC225, are utilized as templates for the generation of an *E. coli* based expression system. The insensitivity to herbicide is tested when the higher plant gene of interest is expressed in a bacterial strain devoid of any endogenous AHAS activity. To this end, the NcoI/PstI fragment of wild type Arabidopsis AHAS (pAC301), the Trp574-Leu substitution (pAC224) and the Trp574 deletion (pAC225) are subcloned into the NcoI/PstI site of the *E. coli* expression vector, pKK233-2 (purchased from Pharmacia). This expression plasmid contains an IPTG inducible promoter as well as a transcription termination sequence. Both regions surround an NcoI, HindIII and PstI linker sequence that allows proper orientation of the gene of interest in relation to the bacterial promoter. Subcloning and ligation of the respective NcoI/PstI fragments into NcoI/PstI digested pKK233-2 DNA (ampicillin resistance) is as previously described. The ligation reaction is transformed into the *E. coli* strain, MF2000 [ilvB800:mu-1, Bgl132, ilv15, thi-1, argE3, RPSL31, (ara-leu, ilvH1) 863, mil-1, xyl-5 galK2, lacY1, recA1]. This bacterial strain is devoid of endogenous AHAS activity. Therefore, growth on a minimal media lacking isoleucine and valine (the end products of amino acid biosynthetic pathway of which the AHAS enzyme is involved) in an MF2000 strain transformed with either pAC224 (Trp574-Leu) or pAC225 (Trp del) requires the expression and function of the Arabidopsis gene in *E. coli*. Each of the three constructions complements MF2000 on agar media as well as in broth cultures.

Single colonies of MF2000 containing either the plasmid construction pAC210, pAC224 or pAC225 are inoculated into 3ml broth cultures of M63 minimal media (amp) containing the amino acids arginine, leucine, isoleucine and valine. [M63 is 30g KH₂PO₄, 70g K₂HPO₄, 20g (NH₄)₂SO₄, 5mg FeSO₄, 100ul of 1M MgSO₄, 200ul thiamine. The glucose concentration is raised to 1.2%]. The cultures are incubated overnight at 37°C with constant shaking. The bacterial cells are pelleted, resuspended in several mls of M63 (amp) supplemented with arginine and leucine, but lacking isoleucine and valine (minus ilv media). The cells are transferred to 10-50 ml of minus ilv media and constant shaking at 37°C was continued overnight. The cells are pelleted and either used directly in AHAS enzyme assays or frozen at -20°C until use.

The extraction and assay for Arabidopsis AHAS in bacterial cells is essentially as described by Singh, et. al. (J. Chromatography 444: 261-267, 1988). The bacterial pellet is powered in liquid nitrogen and homogenized in 100mM potassium phosphate buffer (pH 7.5) containing 10mM pyruvate, 5mM magnesium chloride, 5mM Na₂-EDTA, 100mM flavin adenine dinucleotide (FAD), 1mM valine, 1mM leucine, 10%(v/v) glycerol and 10mM cysteine. The homogenate is filtered through a nylon cloth (53μm mesh) and centrifuged at 25,000 x g for 20 minutes. The supernatant fraction is brought to 50% saturation with respect to ammonium sulfate and allowed to stand for 20-30 minutes on ice. The precipitate is pelleted via centrifugation at 25,000 x g for 20 minutes. The supernatant is discarded and the ammonium sulfate pellet is used immediately or frozen with liquid nitrogen and stored at -20°C until use.

AHAS activity in the presence or absence of herbicide is measured by estimation of the product, acetoacetate, after conversion by acid decarboxylation to acetoin. Standard reaction mixtures contain the

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enzyme (and herbicide) in 50mM potassium phosphate buffer (pH 7.0) containing 100mM sodium pyruvate, 10mM magnesium chloride, 1mM thiamine pyrophosphate (TPP) and 10uM FAD. This mixture is incubated at 37°C for 1 hr. The reaction is stopped with the addition of sulfuric acid to make a final concentration of 0.86%. The reaction product is allowed to decarboxylate at 60°C for 15 minutes. The acetoin formed is determined by incubating with creatine (0.17%) and 1-naphthol (1.7%) by the method of Westerfeld (J. Biol. Chem. 161: 495-502, 1945). Appropriate checks of direct acetoin formation during the enzyme assays are made.

Wild type Arabidopsis AHAS (pAC201) is sensitive to the imidazolinone herbicide Pursuit® and the sulfonylurea herbicide Glean® whereas both the Trp574-Leu substitution (pAC224) and the Trp574 deletion (pAC225) are insensitive to increasing concentrations of Pursuit® herbicide (Figure 4) and Glean® herbicide (Figure 5). Surprisingly, the deletion confers a level of insensitivity to these herbicides which is equivalent or greater than that of the corresponding substitution. Therefore, deletion of the Trp574 residue of the Arabidopsis AHAS coding region results in a functional form of the enzyme that is insensitive to the addition of either of two unrelated herbicides capable of inhibiting the wild type form of the enzyme.

The procedures noted above are also used to create substitution of an asparagine for a serine at position 653, as well as a deletion at this position. Plasmids containing the substitution (pAC229) or the deletion (pAC230) are transformed into *E. coli* MF2000 and AHAS activity of deletion and substitution mutants in the presence of herbicides Pursuit® and Glean® is determined for each as described above. The AHAS produced by substitution of asparagine for serine is insensitive to inhibition by Pursuit® (Figure 6) as previously reported (Sathasivan et. al, *supra*), but sensitivity to Glean® was only slightly reduced (about 10%) as compared with the wild-type enzyme (Figure 7). In contrast, the AHAS produced by the Ser653 deletion is highly resistant to inhibition by both Pursuit® and Glean® (Figures 6 and 7).

DEPOSIT OF BIOLOGICAL MATERIALS

The following microorganisms were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20857, on December 6, 1990, and given accession numbers as follows:

Microorganism	Accession No.
Agrobacterium strain LBA4404 harboring pAC351 (Agro/351) [Trp574 deletion]	68488
Agrobacterium strain LBA4404 harboring plasmid pAC324 [Ser653 deletion]	68489

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Sequence ID No.: 1

Sequence Type: Amino Acid

Sequence Length: 670 Amino Acids

Strandedness: Single

Topology: Linear

Original Source Organism: Arabidopsis

Properties: acetohydroxyacid synthase enzyme

Met Ala Ala Ala Thr Thr Thr Thr Thr Thr Ser Ser
1 5 10

Ser Ile Ser Phe Ser Thr Lys Pro Ser Pro Ser Ser
15 20

Ser Lys Ser Pro Leu Pro Ile Ser Arg Phe Ser Leu
25 30 35

Pro Phe Ser Leu Asn Pro Asn Lys Ser Ser Ser Ser
40 45

Ser Arg Arg Arg Gly Ile Lys Ser Ser Ser Pro Ser
50 55 60

Ser Ile Ser Ala Val Leu Asn Thr Thr Thr Asn Val
65 70

Thr Thr Thr Pro Ser Pro Thr Lys Pro Thr Lys Pro
75 80

Glu Thr Phe Ile Ser Arg Leu Ala Pro Asp Gln Pro
85 90 95

Arg Lys Gly Ala Asp Ile Leu Val Glu Ala Leu Glu
100 105

Arg Gln Gly Val Glu Thr Val Phe Ala Tyr Pro Gly
110 115 120

Gly Ala Ser Met Glu Ile His Gln Ala Leu Thr Arg
125 130

Ser Ser Ser Ile Arg Asn Val Leu Pro Arg His Glu
135 140

Gln Gly Gly Val Phe Ala Ala Glu Gly Tyr Ala Arg
145 150 155

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Ser Ser Gly Lys Pro Gly Ile Cys Ile Ala Thr Ser
 160 165
 Gly Pro Gly Ala Thr Asn Leu Val Ser Gly Leu Ala
 170 175 180
 Asp Ala Leu Leu Asp Ser Val Pro Leu Val Ala Ile
 185 190
 Thr Gly Gln Val Pro Arg Arg Met Ile Gly Thr Asp
 195 200
 Ala Phe Gln Glu Thr Pro Ile Val Glu Val Thr Arg
 205 210 215
 Ser Ile Thr Lys His Asn Tyr Leu Val Met Asp Val
 220 225
 Glu Asp Ile Pro Arg Ile Ile Glu Glu Ala Phe Phe
 230 235 240
 Leu Ala Thr Ser Gly Arg Pro Gly Pro Val Leu Val
 245 250
 Asp Val Pro Lys Asp Ile Gln Gln Gln Leu Ala Ile
 255 260
 Pro Asn Trp Glu Gln Ala Met Arg Leu Pro Gly Tyr
 265 270 275
 Met Ser Arg Met Pro Lys Pro Pro Glu Asp Ser His
 280 285
 Leu Glu Gln Ile Val Arg Leu Ile Ser Glu Ser Lys
 290 295 300
 Lys Pro Val Leu Tyr Val Gly Gly Gly Cys Leu Asn
 305 310
 Ser Ser Asp Glu Leu Gly Arg Phe Val Glu Leu Thr
 315 320
 Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly
 325 330 335
 Ser Tyr Pro Cys Asp Asp Glu Leu Ser Leu His Met
 340 345
 Leu Gly Met His Gly Thr Val Tyr Ala Asn Tyr Ala
 350 355 360
 Val Glu His Ser Asp Leu Leu Leu Ala Phe Gly Val
 365 370

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5 Arg Phe Asp Asp Arg Val Thr Gly Lys Leu Glu Ala
 375 380
 Phe Ala Ser Arg Ala Lys Ile Val His Ile Asp Ile
 385 390 395
 10 Asp Ser Ala Glu Ile Gly Lys Asn Lys Thr Pro His
 400 405
 Val Ser Val Cys Gly Asp Val Lys Leu Ala Leu Gln
 410 415 420
 15 Gly Met Asn Lys Val Leu Glu Asn Arg Ala Glu Glu
 425 430
 Leu Lys Leu Asp Phe Gly Val Trp Arg Asn Glu Leu
 435 440
 20 Asn Val Gln Lys Gln Lys Phe Pro Leu Ser Phe Lys
 445 450 455
 Thr Phe Gly Glu Ala Ile Pro Pro Glu Tyr Ala Ile
 460 465
 25 Lys Val Leu Asp Glu Leu Thr Asp Gly Lys Ala Ile
 470 475 480
 30 Ile Ser Thr Gly Val Gly Gln His Gln Met Trp Ala
 485 490
 Ala Gln Phe Tyr Asn Tyr Lys Lys Pro Arg Gln Trp
 495 500
 35 Leu Ser Ser Gly Gly Leu Gly Ala Met Gly Phe Gly
 505 510 515
 40 Leu Pro Ala Ala Ile Gly Ala Ser Val Ala Asn Pro
 520 525
 Asp Ala Ile Val Val Asp Ile Asp Gly Asp Gly Ser
 530 535 540
 45 Phe Ile Met Asn Val Gln Glu Leu Ala Thr Ile Arg
 545 550
 Val Glu Asn Leu Pro Val Lys Val Leu Leu Leu Asn
 555 560
 50 Asn Gln His Leu Gly Met Val Met Gln Trp Glu Asp
 565 570 575
 55 Arg Phe Tyr Lys Ala Asn Arg Ala His Thr Phe Leu
 580 585

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5 Gly Asp Pro Ala Gln Glu Asp Glu Ile Phe Pro Asn
590 595 600

Met Leu Leu Phe Ala Ala Ala Cys Gly Ile Pro Ala
605 610

10 Ala Arg Val Thr Lys Lys Ala Asp Leu Arg Glu Ala
615 620

Ile Gln Thr Met Leu Asp Thr Pro Gly Pro Tyr Leu
625 630 635

15 Leu Asp Val Ile Cys Pro His Gln Glu His Val Leu
640 645

20 Pro Met Ile Pro Ser Gly Gly Thr Phe Asn Asp Val
650 655 660

Ile Thr Glu Gly Asp Gly Arg Ile Lys Tyr
665 670

25

30

35

40

45

50

55

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Sequence ID No.: 2

Sequence Type: Nucleic Acid

5 Sequence Length: 2,526 Base Pairs

Strandedness: Single

10 Topology: Linear

Original Source Organism: Arabidopsis75 Properties: gene encoding acetohydroxyacid synthase
enzyme

NTAAAGAAA GAAAGAAAGA TCAATTGAT AAATTTCTCA 40
GCCACAAAT CTACATTTAG GTTTTAGCAT ATCGAAGGCT 80
CAATCAGAA TACAATAGAT AGACTAGAGA TTCCAGCGTC 120
ACGTGAGTTT TATCTATAAA TAAAGGACCA AAAATCAAA 160
25 CCCGAGGGCA TTTTCGTAA CCAACATATA ACCCTTAAAC 200
TTCAAGTCTC ATTTTTAAAC AAATCATGTT CACAAGTCTC 240
TTCTTCTTCT CTGTTTCTCT ATCTCTTGCT CATCTTTCTC 280
30 CTGAACCATG GCGGCGGCAA CAACAACAAC AACAACATCT 320
TCTTCGATCT CCTTCTCCAC CAACCATCT CCTTCCTCCT 360
35 CCAAAATCAC ATTACCAATC TCCAGATTCT CCTTCCCATT 400
CTCCCTAAAC CCCAACAAAT CATCCTCCTC CTCCCGCCGC 440
CGCGGTATCA AATCCAGCTC TCCCTCCTCC ATCTCCGCCG 480
40 TGCTCAACAC AACCACCAAT GTCACAACCA CTCCCTCTCC 520
AACCAAACCT ACCAAACCCG AAACATTCTT CTCCCGATTG 560
45 GCTCCAGATC AACCCCGCAA AGGCGCTGAT ATCCTCGTCG 600
AAGCTTTAGA ACGTCAAGGC GTAGAAACCG TATTGGCTTA 640
CCCTGGAGGT GCATCAATGG AGATTACCA AGCCTTAACC 680
50 CGCTCTTCCT CAATCCGTAA CGTCCTTCCT CGTCACGAA 720
AAGGAGGTGT ATTCGCAGCA GAAGGATACG CTCGATCCTC 760
65 AGGTAAACCA GGTATCTGTA TAGCCACTTC AGGTCCCGGA 800

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	GCTACAAATC TCGTTAGCGG ATTAGCCGAT GCGTTGTTAG	840
5	ATAGTGTTC TCTGTAGCA ATCAGAGGAC AAGTCCCTCG	880
	TCGTATGATT GGTACAGATG CGTTTCAAGA GACTCCGATT	920
10	GTTGAGGTAA CGCGTTCGAT TACGAAGCAT AACTATCTTG	960
	TGATGGATGT TGAAGATATC CCTAGGATTA TTGAGGAAGC	1000
	TTTCTTTTTA GCTACTTCTG GTAGACCTGG ACCTGTTTTG	1040
15	GTTGATGTTT CTAAAGATAT TCAACAACAG CTTGCCGATT	1080
	CTAATTGGGA ACAGGCTATG AGATTACCTG GTTATATGTC	1120
	TAGGATGCCCT AAACCTCCGG AAGATTCTCA TTTGGAGCAG	1160
20	ATTGTTAGGT TGATTTCTGA GTCTAAGAAG CCTGTGTTGT	1200
	ATGTTGGTGG TGATTGTTT AATCTAGCG ATGAATTGGG	1240
25	TAGGTTTGTG GAGCTTACGG GGATCCCTGT TGCAGGTACG	1280
	TTGATGGGGC TGGGATCTTA TCCTTGATGAT GATGAGTTGT	1320
	CGTTACATAT GCTTGGAAATG CATGGGACTG TGTATGCAA	1360
30	TTACGCTGTG GAGCATAGTG ATTTGTTGTT GCGGTTTGGG	1400
	GTAAGGTTTG ATGATCGTGT CACGGGTAAAG CTTGAGGCTT	1440
35	TTGCTAGTAG GGCTAAGATT GTTCATATTG ATATTGACTC	1480
	GGCTGAGATT GGGAAAGAATA AGACTCCTCA TGTGTCTGTG	1520
	TGTGGTGATG TTAAGCTGGC TTTGCAAGGG ATGAATAAGG	1560
40	TTCTTGAGAA CCGAGCGGAG GAGCTTAAGC TTGATTTTGG	1600
	AGTTTGGAGG AATGAGTTGA ACGTACAGAA ACAGAAGTTT	1640
45	CCGTTGAGCT TTAAGACGTT TGGGGAAAGCT ATTCCCTCCAC	1680
	AGTATGCGAT TAAGGTCTT GATGAGTTGA CTGATGGAAA	1720
	AGCCATAATA AGTACTGGTG TCGGGCAACA TCAAATGTGG	1760
50	GCGGCGCAGT TCTACAATTA CAAGAAACCA AGGCAGTGGC	1800
	TATCATCAGG AGGCCTTGGG GCTATGGGAT TTGGACTTCC	1840
55	TGCTGCGATT GGAGCGTCTG TTGCTAACCC TGATGCGATA	1880

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5 GTTGTGGATA TTGACGGAGA TGGAAAGCTTT ATAATGAATG 1920
 TGCAAGAGCT AGCCACTATT CQTGTAGAGA ATCTTCCAGT 1960
 GAAGGTACTT TTATTAAACA ACCAGCATCT TGGCATGGTT 2000
 10 ATGCAATGGG AAGATCGGTT CTACAAAGCT AACCGAGCTC 2040
 ACACATTTCT CGGGGATCCG GCTCAGGAGG ACGAGATATT 2080
 CCCGAACATG TTGCTGTTTG CAGCAGCTTG CGGGATTCCA 2120
 15 GCGGCGAGGG TGACAAAGAA AGCAGATCTC CGAGAAGCTA 2160
 TTCAGACAAT GCTGGATACA CCAGGACCTT ACCTGTTGGA 2200
 TGTGATTTGT CCGCACCAAG AACATGTGTT GCCGATGATC 2240
 20 CCGAGTGGTG GCACTTTCAA CGATGTGATA ACGGAAGGAG 2280
 ATGGCCGGAT TAAATACTGA GAGATGAAAC CGGTGATTAT 2320
 25 CAGAACCCTT TATGGTCTTT GTATGCATAT GGTAAAAAAA 2360
 CTTAGTTTGC AATTTCCTGT TTGTTTTGGT AATTTGAGTT 2400
 TCTTTTAGTT GTTGATCTGC CTGCTTTTGG GTTTACGTCG 2440
 30 GACTACTACT GCTGTTGTTG TTTGGTTTCC TTTCTTTCAT 2480
 TTTATAAATA AATAATCCGG TTCGGTTTAC TCCTTGTGAC 2520
 35 TGGCTC 2526

40 Claims

1. A method for expressing an herbicide resistant AHAS enzyme in a host cell comprising transforming the host cell with a nucleic acid sequence encoding a functional AHAS enzyme, which enzyme has an amino acid sequence differing from a wild-type AHAS sequence in having at least one amino acid deletion that confers herbicide resistance to the enzyme, and culturing the host cell under conditions permitting expression of the enzyme.
2. The method of Claim 1 in which a deletion is present in a conserved region of the AHAS sequence.
3. The method of any of Claims 1-2 in which a deletion has occurred in a region of the wild type AHAS sequence *Arabidopsis* selected from the group consisting of amino acids 110-122, 184-187, 201-208, 255-257, 348-353, 373-377, 569-578, and 650-653, or the homologous regions in a different species.
4. The method of Claims 1-3 in which the deletion occurs at at least one position selected from the group consisting of amino acids 121, 122, 187, 205, 256, 351, 378, 571, 574, 578 and 653.
5. The method of any of Claims 1-4 in which resistance is to at least one herbicide selected from the group consisting of an imidazolinone, a sulfonylurea and a triazolopyrimidine.

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6. The method of any of Claims 1-5 in which the host cell is a plant cell.
7. The method of Claim 6 in which the transformed plant cell is regenerated to a mature plant expressing an herbicide resistant phenotype.
8. A method for producing a vector useful in conferring herbicide resistance to a host cell comprising ligating into an appropriate vector a nucleic acid sequence encoding a functional AHAS enzyme, which enzyme has an amino acid sequence differing from a wild type AHAS in having at least one amino acid deletion that confers herbicide resistance to the enzyme.
9. A method for conferring herbicide resistance to a plant cell which comprises providing the plant cell with a nucleic acid sequence encoding a functional AHAS enzyme, which enzyme has an amino acid sequence differing from a wild type AHAS sequence in having at least one amino acid deletion that confers herbicide resistance to the enzyme.
10. A method for growing herbicide resistant plants which comprises cultivating a plant which produces a functional AHAS enzyme with an amino acid sequence differing from a wild type AHAS sequence in having at least one amino acid deletion that confers herbicide resistance to the enzyme, in the presence of an inhibitory amount of the herbicide.
11. A method of selecting host cells successfully transformed with a gene of interest which comprises providing to prospective host cells the gene of interest linked to a nucleic acid sequence encoding a functional AHAS enzyme, which enzyme has an amino acid sequence differing from a wild-type AHAS sequence in having at least one amino acid deletion that confers herbicide resistance to the enzyme, and culturing the host cell under conditions permitting expression of the enzyme, growing the cells in the presence of an inhibitory amount of a herbicide, and identifying surviving cells as containing the gene of interest.

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Met Ala Ala Ala Thr Thr Thr Thr Thr Thr Ser Ser
 1 5 10
 Ser Ile Ser Phe Ser Thr Lys Pro Ser Pro Ser Ser
 15 20
 Ser Lys Ser Pro Leu Pro Ile Ser Arg Phe Ser Leu
 25 30 35
 Pro Phe Ser Leu Asn Pro Asn Lys Ser Ser Ser Ser
 40 45
 Ser Arg Arg Arg Gly Ile Lys Ser Ser Ser Pro Ser
 50 55 60
 Ser Ile Ser Ala Val Leu Asn Thr Thr Thr Asn Val
 65 70
 Thr Thr Thr Pro Ser Pro Thr Lys Pro Thr Lys Pro
 75 80
 Glu Thr Phe Ile Ser Arg Leu Ala Pro Asp Gln Pro
 85 90 95
 Arg Lys Gly Ala Asp Ile Leu Val Glu Ala Leu Glu
 100 105
 Arg Gln Gly Val Glu Thr Val Phe Ala Tyr Pro Gly
 110 115 120
 Gly Ala Ser Met Glu Ile His Gln Ala Leu Thr Arg
 125 130
 Ser Ser Ser Ile Arg Asn Val Leu Pro Arg His Glu
 135 140
 Gln Gly Gly Val Phe Ala Ala Glu Gly Tyr Ala Arg
 145 150 155
 Ser Ser Gly Lys Pro Gly Ile Cys Ile Ala Thr Ser
 160 165
 Gly Pro Gly Ala Thr Asn Leu Val Ser Gly Leu Ala
 170 175 180
 Asp Ala Leu Leu Asp Ser Val Pro Leu Val Ala Ile
 185 190
 Thr Gly Gln Val Pro Arg Arg Met Ile Gly Thr Asp
 195 200
 Ala Phe Gln Glu Thr Pro Ile Val Glu Val Thr Arg
 205 210 215

FIG.1a(i)

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Ser Ile Thr Lys His Asn Tyr Leu Val Met Asp Val
 220 225
 Glu Asp Ile Pro Arg Ile Ile Glu Glu Ala Phe Phe
 230 235 240
 Leu Ala Thr Ser Gly Arg Pro Gly Pro Val Leu Val
 245 250
 Asp Val Pro (Lys) Asp Ile Gln Gln Gln Leu Ala Ile
 255 260
 Pro Asn Trp Glu Gln Ala Met Arg Leu Pro Gly Tyr
 265 270 275
 Met Ser Arg Met Pro Lys Pro Pro Glu Asp Ser His
 280 285
 Leu Glu Gln Ile Val Arg Leu Ile Ser Glu Ser Lys
 290 295 300
 Lys Pro Val Leu Tyr Val Gly Gly Gly Cys Leu Asn
 305 310
 Ser Ser Asp Glu Leu Gly Arg Phe Val Glu Leu Thr
 315 320
 Gly Ile Pro Val Ala Ser Thr Leu Met Gly Leu Gly
 325 330 335
 Ser Tyr Pro Cys Asp Asp Glu Leu Ser Leu His Met
 340 345
Leu Gly (Met) His Gly Thr Val Tyr Ala Asn Tyr Ala
 350 355 360
 Val Glu His Ser Asp Leu Leu Leu Ala Phe Gly Val
 365 370
Arg Phe Asp (Asp) Arg Val Thr Gly Lys Leu Glu Ala
 375 380
 Phe Ala Ser Arg Ala Lys Ile Val His Ile Asp Ile
 385 390 395
 Asp Ser Ala Glu Ile Gly Lys Asn Lys Thr Pro His
 400 405
 Val Ser Val Cys Gly Asp Val Lys Leu Ala Leu Gln
 410 415 420
 Gly Met Asn Lys Val Leu Glu Asn Arg Ala Glu Glu
 425 430

FIG.1a(ii)

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Leu Lys Leu Asp Phe Gly Val Trp Arg Asn Glu Leu
 435 440
 Asn Val Gln Lys Gln Lys Phe Pro Leu Ser Phe Lys
 445 450 455
 Thr Phe Gly Glu Ala Ile Pro Pro Gln Tyr Ala Ile
 460 465
 Lys Val Leu Asp Glu Leu Thr Asp Gly Lys Ala Ile
 470 475 480
 Ile Ser Thr Gly Val Gly Gln His Gln Met Trp Ala
 485 490
 Ala Gln Phe Tyr Asn Tyr Lys Lys Pro Arg Gln Trp
 495 500
 Leu Ser Ser Gly Gly Leu Gly Ala Met Gly Phe Gly
 505 510 515
 Leu Pro Ala Ala Ile Gly Ala Ser Val Ala Asn Pro
 520 525
 Asp Ala Ile Val Val Asp Ile Asp Gly Asp Gly Ser
 530 535 540
 Phe Ile Met Asn Val Gln Glu Leu Ala Thr Ile Arg
 545 550
 Val Glu Asn Leu Pro Val Lys Val Leu Leu Leu Asn
 555 560
 Asn Gln His Leu Gly Met (Val) Met Gln (Trp) Glu Asp
 565 570 575
 Arg (Phe) Tyr Lys Ala Asn Arg Ala His Thr Phe Leu
 580 585
 Gly Asp Pro Ala Gln Glu Asp Glu Ile Phe Pro Asn
 590 595 600
 Met Leu Leu Phe Ala Ala Ala Cys Gly Ile Pro Ala
 605 610
 Ala Arg Val Thr Lys Lys Ala Asp Leu Arg Glu Ala
 615 620
 Ile Gln Thr Met Leu Asp Thr Pro Gly Pro Tyr Leu
 625 630 635
 Leu Asp Val Ile Cys Pro His Gln Glu His Val Leu
 640 645

FIG. 1a(iii)

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Pro	Met	Ile	Pro	Ser	Gly	Gly	Thr	Pha	Asn	Asp	Val
	650				655					660	
Ile	Thr	Glu	Gly	Asp	Gly	Arg	Ile	Lys	Tyr		
			665						670		

FIG.1a(iiii)

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NTRAAARAAA GAAAGAAAGA TCAATTTGAT AAATTTCTCA	40
GCCACAAATT CTACATTTAG GTTTTAGCAT ATCGAAGGCT	80
CAATCACAAA TACAATAGAT AGACTAGAGA TTCCAGCGTC	120
ACGTGAGTTT TATCTATAAA TAAAGGACCA AAAATCAAAT	160
CCCGAGGGCA TTTCGTAAT CCAACATAAA ACCCTTAAAC	200
TTCAAGTCTC ATTTTAAAC AAATCATGTT CACAAGTCTC	240
TTCTTCTTCT CTGTTTCTCT ATCTCTTGCT CATCTTTCTC	280
CTGAACCATG GCGGCGGCAA CAACAACAAC AACAACATCT	320
TCTTCGATCT CTTCTCCAC CAAACCATCT CTTCTCTCT	360
CCAAATCACC ATTACCAATC TCCAGATTCT CCTTCCCATT	400
CTCCCTAAAC CCCAACAAAT CATCTCTCTC CTCCCGCCGC	440
CGCGGTATCA AATCCAGCTC TCCCTCTCTC ATCTCCGCGC	480
TGCTCAACAC AACCACCAAT GTCACAACCA CTCCCTCTCT	520
AAACCAACCT ACCAAACCCG AAACATTCAT CTCCCGATTG	560
GCTCCAGATC AACCCCGCAA AGGCGGTGAT ATCCTCGTCC	600
AAGCTTTAGA ACGTCAAGGC GTAGAAACCG TATTGCTTA	640
CCCTGGAGGT GCATCAATGG AGATTACCA AGCCTTAACC	680
CGCTCTTCTT CAATCCGTAA CGTCTTCTCT CGTCACGAAC	720
AAGGAGGTGT ATTGCGAGCA GAAGGATACG CTCGATCCTC	760
AGGTAAACCA GGTATCTGTA TAGCCACTTC AGGTCCCGGA	800
GCTACAAATC TCGTTAGCGG ATTAGCCGAT GCGTTGTTAG	840
ATAGTGTTCC TCTTGAGCA ATCACAGGAC AAGTCCCTCG	880
TCGTATGATT GGTACAGATG CGTTTCAAGA GACTCCGATT	920
GTTGAGGTAA CGCGTTGAT TACGAAGCAT AACTATCTTG	960
TGATGGATGT TGAAGATATC CCTAGGATTA TTGAGGAAGC	1000
TTTCTTTTAA GCTACTTCTG GTAGACCTGG ACCTGTTTTG	1040
GTTGATGTTT CTAAAGATAT TCAACAACAG CTGCGATTTC	1080

FIG.1b(i)

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CTAATTGGGA ACAGGCTATG AGATTACCTG GTTATATGTC	1120
TAGGATGCCT AAACCTCCGG AAGATTCTCA TTTGGAGCAG	1160
ATTGTTAGGT TGATTTCTGA GTCTAAGAAG CCTGTGTTGT	1200
ATGTTGGTGG TGGTTGTTTG AATTCTAGCG ATGAATTGGG	1240
TAGGTTTGTG GAGCTTACGG GGATCCCTGT TGGAGTACG	1280
TTGATGGGGC TGGGATCTTA TCCTTGTGAT GATGAGTTGT	1320
CGTTACATAT GCTTGGAAAG CATGGGACTG TGTATGCAA	1360
TTACGCTGTG GAGCATACTG ATTTGTTGTT GCGTTTGGG	1400
GTAAGGTTTG ATGATCGTGT CACGGGTAAG CTTGAGGCTT	1440
TTGCTAGTAG GGCTAAGATT GTTCATATTG ATATTGACTC	1480
GGCTGAGATT GCGAAGAATA AGACTCCTCA TGTGCTGTGT	1520
TGTGGTGATG TTAAGCTGGC TTTGCAAGGG ATGAATAAGG	1560
TTCTTGAGAA CCGAGCCGAG GAGCTTAAGC TTGATTTTGG	1600
AGTTTGGAGG AATGACTTGA ACGTACAGAA ACAGAAGTTT	1640
CCGTTGAGCT TTAAGACCTT TGGGGAAGCT ATTCCTCCAC	1680
AGTATGCGAT TAAGGTCCTT GATGAGTTGA CTGATGGAAA	1720
AGCCATAATA AGTACTGGTG TCGGGCAACA TCAAATGTGG	1760
GCGGCGCAGT TCTACAATTA CAAGAAACCA AGGCAGTGGC	1800
TATCATCAGG AGGCCTTGGG GCTATGGGAT TTGGACTTCC	1840
TGCTGCGATT GGAGCGTCTG TTGCTAACCC TGATGCGATA	1880
GTTGTGGATA TTGACGGAGA TGGAAAGCTT ATAATGAATG	1920
TGCAAGAGCT AGCCACTATT CGTGTAGAGA ATCTTCCAGT	1960
GAAGGTAATT TTATTAAACA ACCAGCATCT TGGCATGCTT	2000
ATGCAANTGGG AAGATCGGTT CTACAAAGCT AACCGAGCTC	2040
ACACATTTCT CGGGGATCCG GCTCAGGAGG ACCAGATATT	2080
CCCGAACATG TTGCTGTTTG CAGCAGCTTG CGGGATTCCA	2120
GCGGCGAGGG TGACAAAGAA AGCAGATCTC CGAGAAGCTA	2160

FIG.1b(ii)

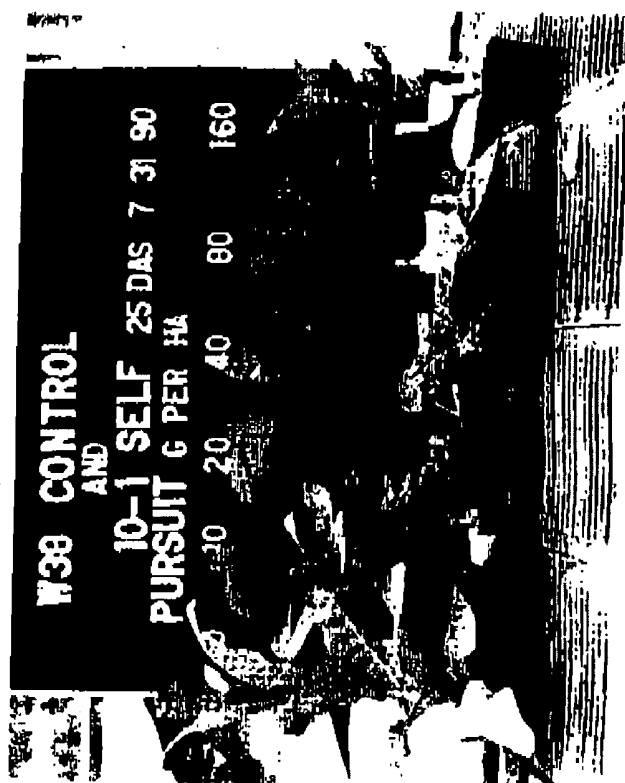
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TTCAGACAAT GCTGGATACA CCAGGACCTT ACCTGTTGGA	2200
TGTGATTTGT CCGCACCAAG AACATGTGTT GCCGATCATC	2240
CCGAGTGGTG GCACTTTCAA CGATGTCATA ACGGAAGGAC	2280
ATGGCCGGAT TAAATACTGA GAGATGAAAC CGGTGATTAT	2320
CAGAACCTTT TATGCTCTTT GTATGCATAT GGTAACAAAA	2360
CTTAGTTTGC AATTTCTGT TTGTTTGGT AATTTGAGTT	2400
TCTTTTAGTT GTTGATCTGC CTGCTTTTG GTTTACGTCA	2440
GACTACTACT GCTGTTGTTG TTGCTTTCC TTTCTTTCAT	2480
TTTATAAATA AATAATCCGG TTCGGTTTAC TCCTTGTGAC	2520
TGGCTC	2526

FIG.1b(iii)

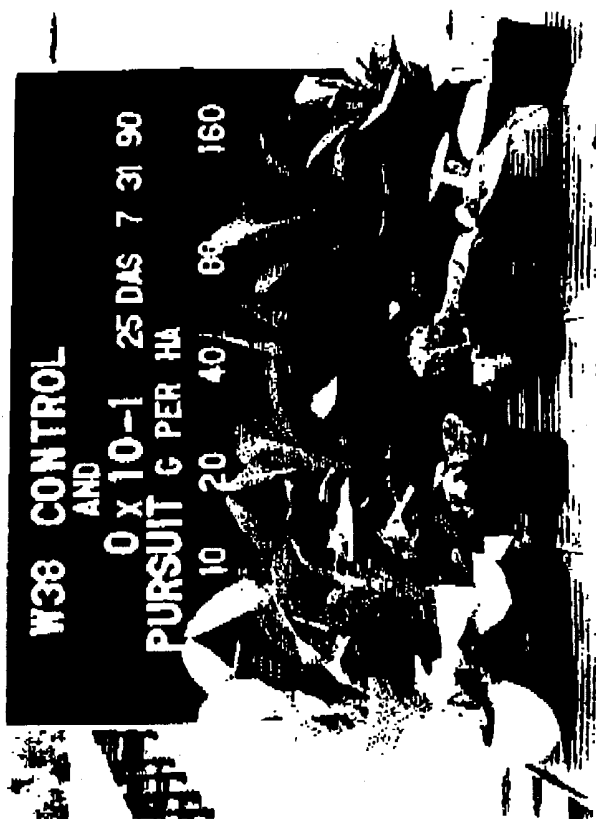
EP 0 482 113 A2

FIG. 2a



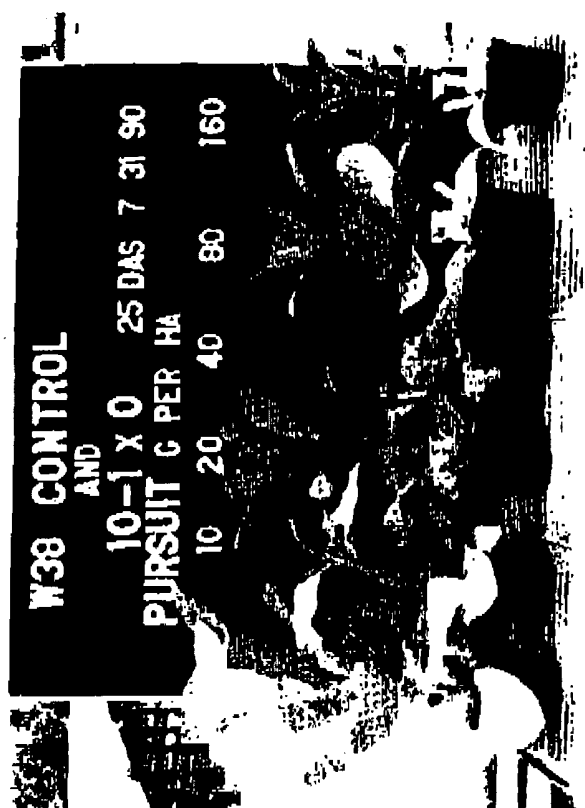
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FIG. 2b



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FIG.2c



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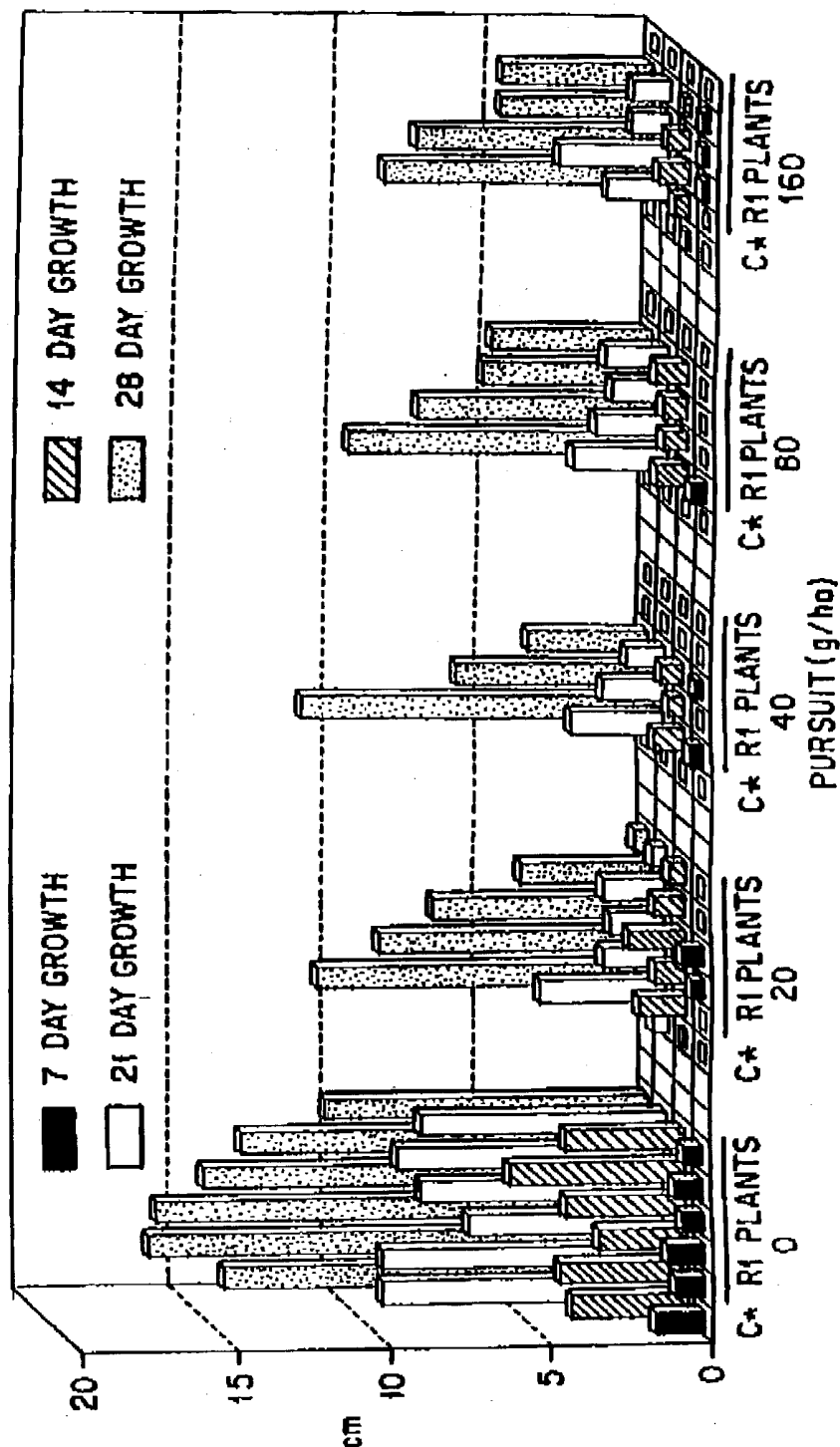


FIG. 3

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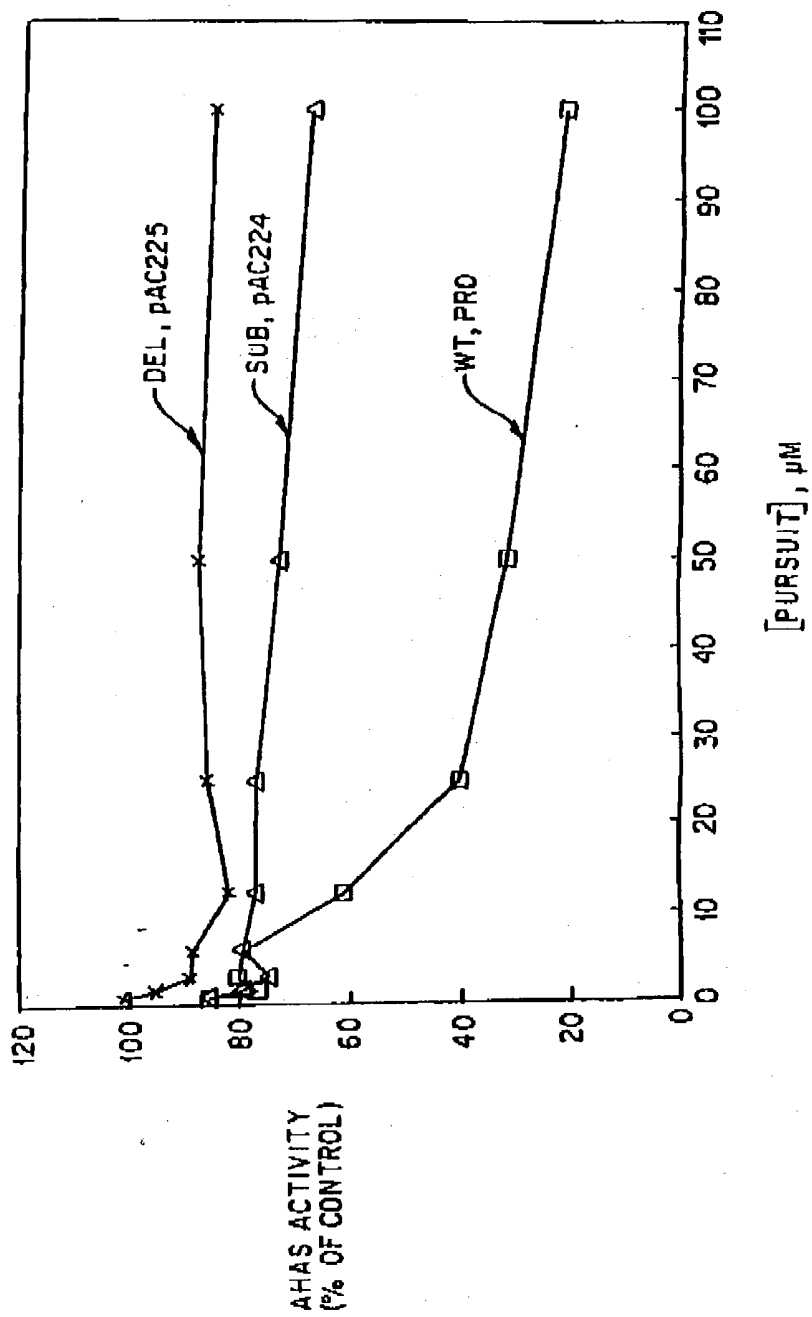


FIG. 4

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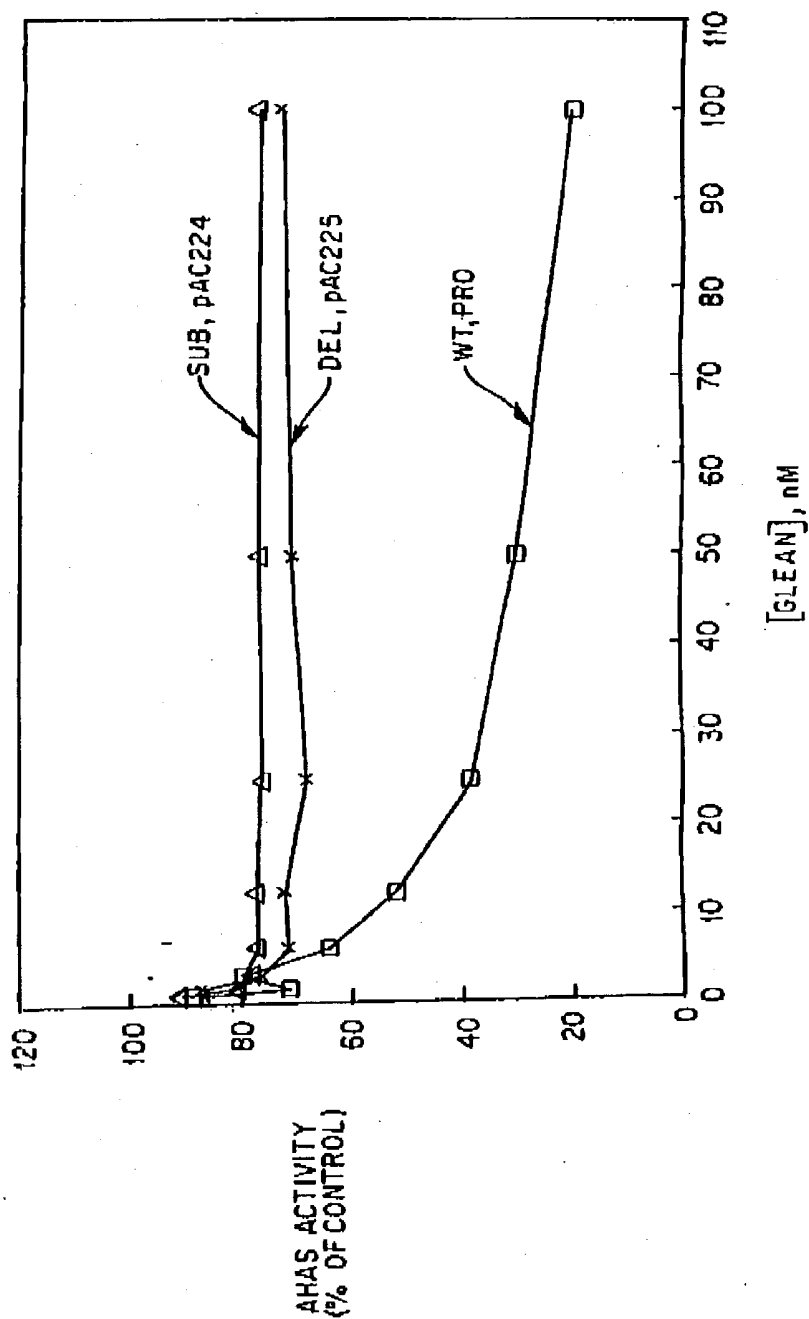


FIG. 5

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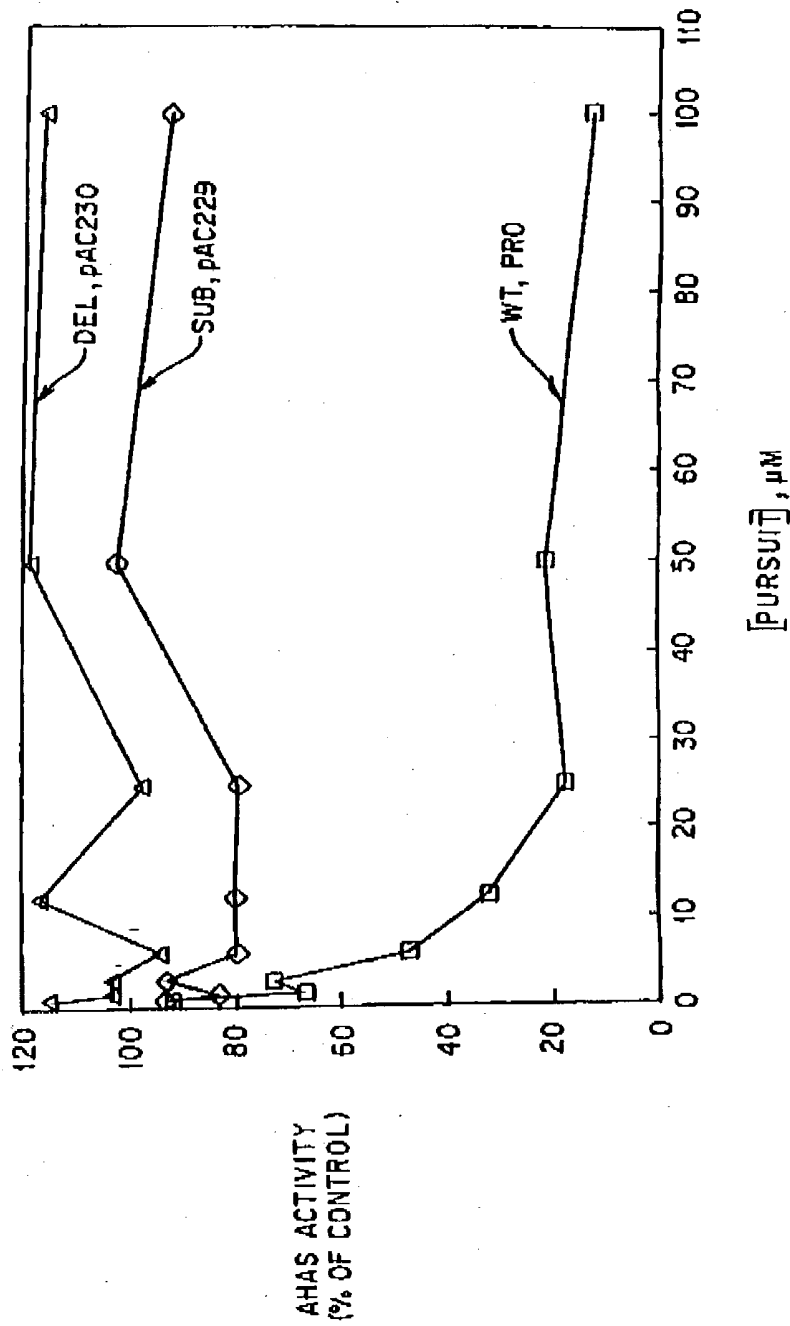


FIG. 6

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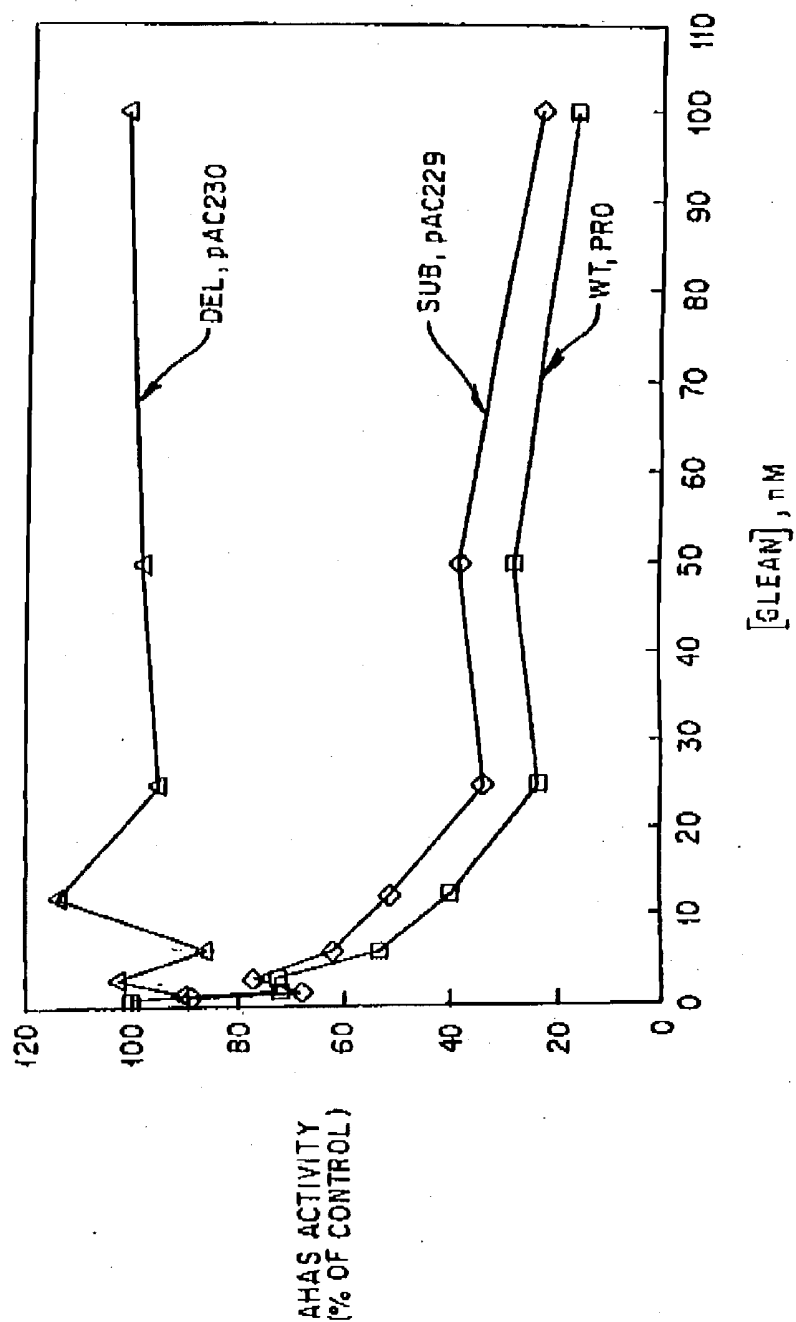


FIG. 7

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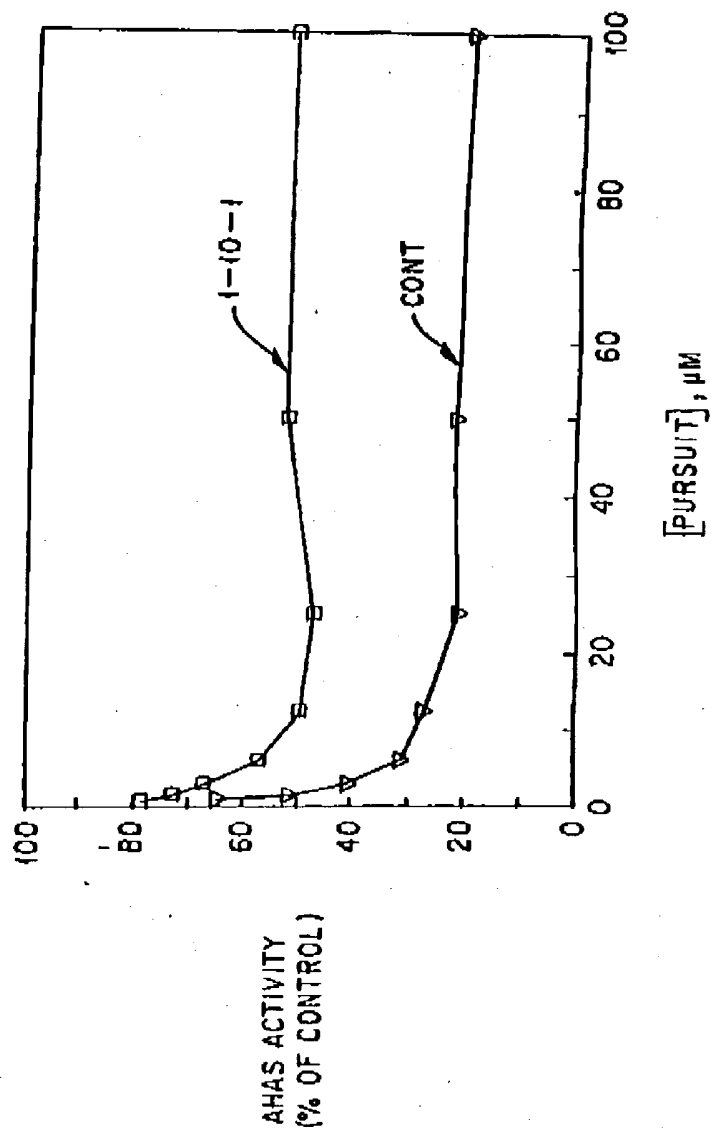


FIG. 8